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[54] **IN VITRO PROPAGATION OF EMBRYONIC STEM CELLS**

[75] Inventors: **Robert L. Williams**, Warrándyte; **Nicholas M. Gough**, North Balwyn; **Douglas J. Hilton**, Warrandyte, all of Australia

[73] Assignee: **Amrad Corporation Limited**, Kew, Australia

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[52] **U.S. Cl.** **435/240.1**; 435/240.2; 435/240.3

[58] **Field of Search** 435/240.1, 240.2, 240.3

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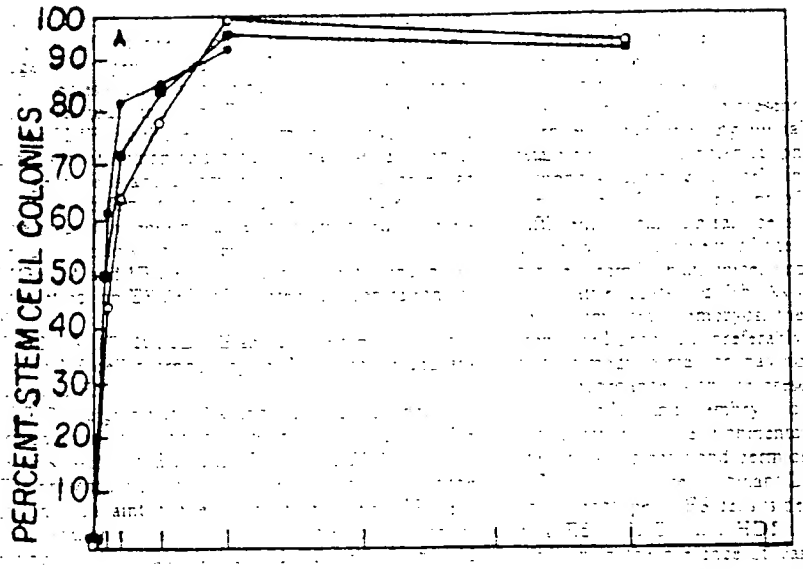
Primary Examiner—John J. Doll
Assistant Examiner—George C. Elliott
Attorney, Agent, or Firm—Iver P. Cooper

[57] **ABSTRACT**

The present invention relates generally to the use of leukaemia inhibitory factor (LIF) in the maintenance and derivation of embryonic stem (ES) cells in culture. The ES cells are maintained and/or derived from animal embryos by culturing said cells or embryos in a culture medium containing an effective amount of LIF for a time and under conditions sufficient to maintain and/or derive said ES cells. The ES cells may be passaged in LIF and used to make chimaeric animals.

16 Claims, 4 Drawing Sheets

FIG 1A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100
90
80
70
60
50
40
30
20
10

FIG 1B

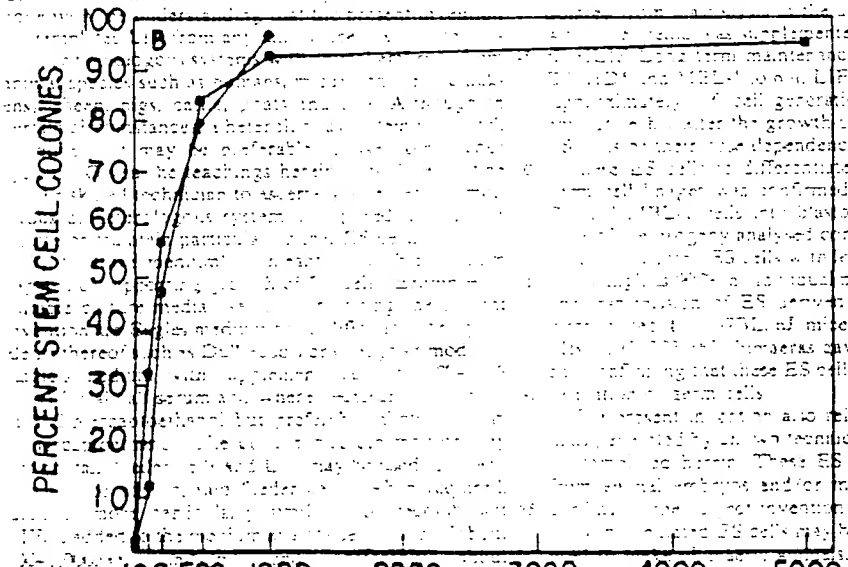

B
PERCENT STEM CELL COLONIES
100
90
80
70
60
50
40
30
20
10
100 500 1000 2000 3000 4000 5000
LIF (UNITS/ml)

FIG. 2A

FIG. 2B

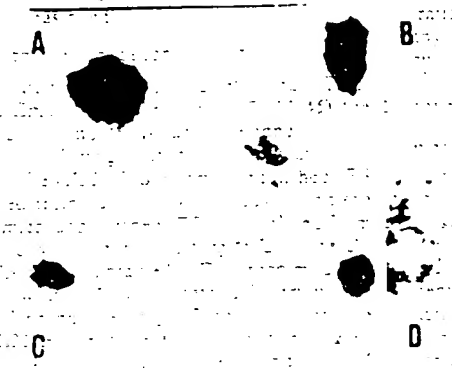


FIG. 2C

FIG. 2D

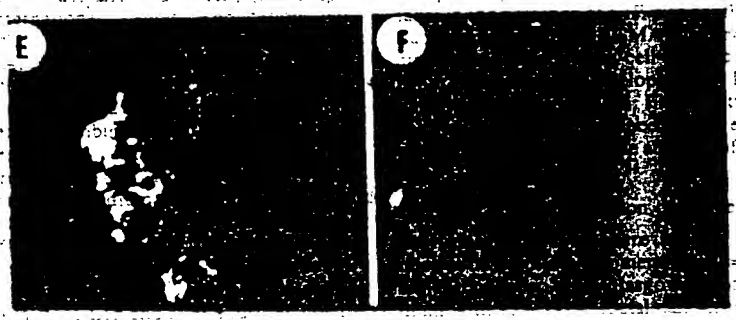
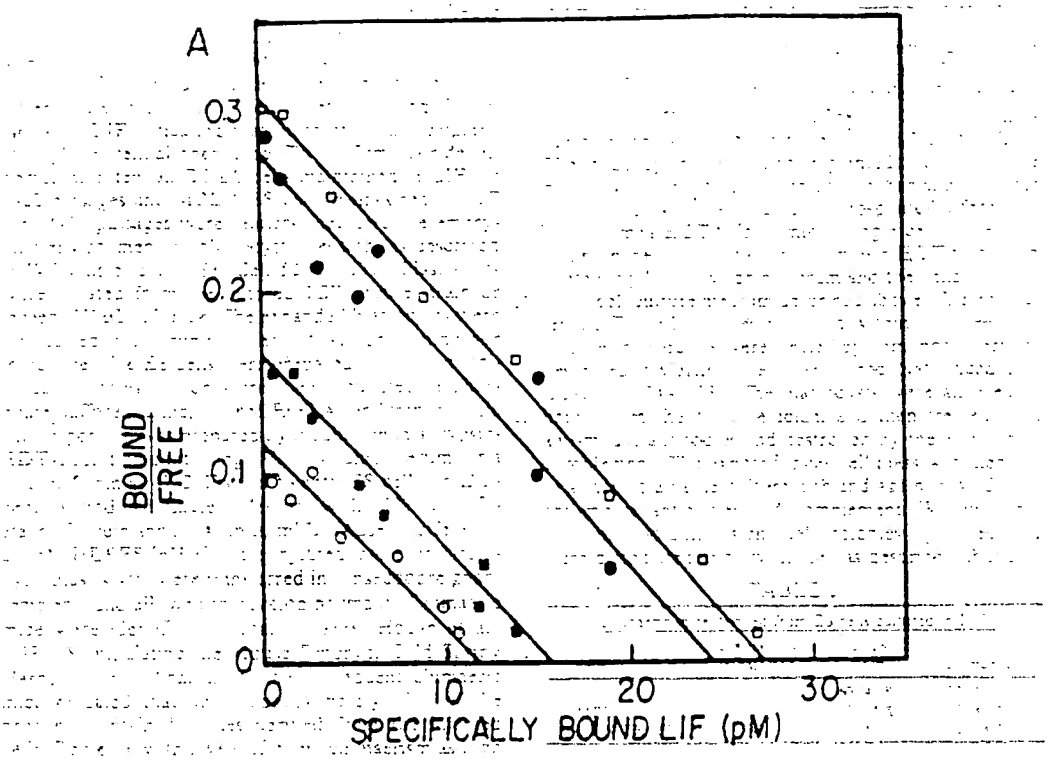


FIG. 2E

FIG. 2F

FIG. 3A


A
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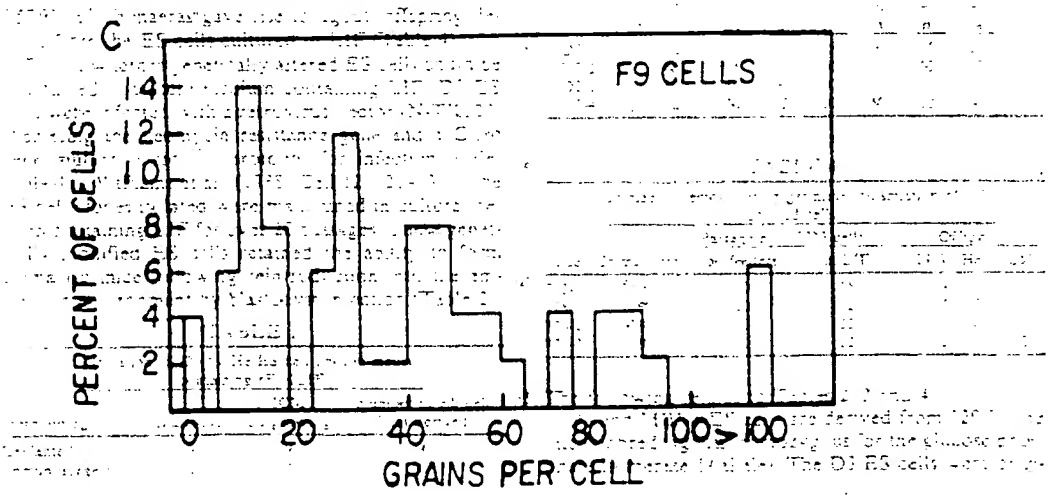
0
BOUND
FREE
0
10
20
30
SPECIFICALLY BOUND LIF (pM)

FIG. 3C


C
F9 CELLS
PERCENT OF CELLS
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12
10
8
6
4
2
0
20
40
60
80
100
>100
GRAINS PER CELL

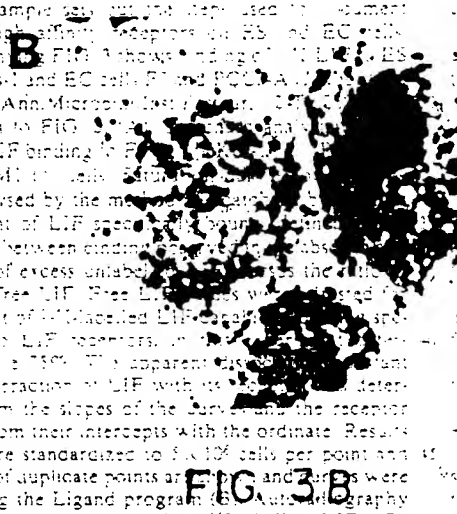

FIG. 3B

IN VITRO PROPAGATION OF EMBRYONIC STEM CELLS

This invention relates to the use of a previously discovered and characterised molecule, leukaemia inhibitory factor (LIF), in the isolation and propagation of embryonic stem cells in vitro.

Embryonic stem (ES) cells, the pluripotent outgrowths of blastocysts, can be cultured and manipulated in vitro and then returned to the embryonic environment to contribute normally to all tissues including the germline (for review see Robertson, E. J. (1986) Trends in Genetics 2:9–13). Not only can ES cells propagated in vitro contribute efficiently to the formation of chimaeras, including germline chimaeras, but in addition, these cells can be manipulated in vitro without losing their capacity to generate germ-line chimaeras (Robertson, E. J. et. al. (1986) Nature 323:445–447).

ES cells thus provide a route for the generation of transgenic animals such as transgenic mice, a route which has a number of important advantages compared with more conventional techniques, such as zygote injection and viral infection (Wagner and Stewart (1986) in Experimental Approaches to Embryonic Development. J. Rossant and A. Pedersen eds. Cambridge: Cambridge University Press), for introducing new genetic material into such animals. First, the gene of interest can be introduced and its integration and expression characterised in vitro. Secondly, the effect of the introduced gene on the ES cell growth can be studied in vitro. Thirdly, the characterised ES cells having a novel introduced gene can be efficiently introduced into embryos by blastocyst injection or embryo aggregation and the consequences of the introduced gene on the development of the resulting transgenic chimaeras monitored during pre- or post-natal life. Fourthly, the site in the ES cell genome at which the introduced gene integrates can be manipulated, leaving the way open for gene targeting and gene replacement (Thomas, K. R. and Capecci, M. R. (1987) Cell 51:503–512).

However, it is known that ES cells and certain EC (embryonal carcinoma) cell lines will only retain the stem cell phenotype in vitro when cultured on a feeder layer of fibroblasts (such as murine STO cells, e.g. Martin, G. R. and Evans, M. J. (1975) Proc. Natl. Acad. Sci. USA 72:1441–1445) or when cultured in medium conditioned by certain cells (e.g. Koopman, P. and Cotton, R. G. H. (1984) Exp. Cell Res. 154:233–242; Smith, A. G. and Hooper, M. L. (1987) Devel.Biol. 121:1–91). In the absence of feeder cells or conditioned medium, the ES cells spontaneously differentiate into a wide variety of cell types, resembling those found during embryogenesis and in the adult animal. The factors responsible for maintaining the pluripotency of ES cells have, however, remained poorly characterised.

In work leading to the present invention, it has been found that LIF has the capacity to substitute for, or be added to, feeder layers (or conditioned medium) in supporting the maintenance of pluripotential ES cells in vitro.

LIF is a protein that has previously been purified, cloned and produced in large quantities in purified recombinant form from both *Escherichia coli* and yeast cells. (International Patent Application No. PCT/AU88/00093, filed Mar. 31, 1988.) LIF has been defined as a factor, the properties of which include:

1. it has the ability to suppress the proliferation of myeloid leukaemic cells such as M1 cells, with associated differentiation of the leukaemic cells; and

2. it will compete with a molecule having the defined sequence of murine LIF or human LIF (defined in International Patent Application No. PCT/AU88/00093) for binding to specific cellular receptors on M1 cells or murine or human macrophages. In addition to the biological properties previously disclosed for murine and human LIF, LIF has now been found to have the following properties:

(a) it allows the derivation and maintenance in the absence of feeder cells of the pluripotential phenotype in vitro of ES cells.

(b) it allows the aforementioned ES cells, after passage in vitro in the presence of LIF, to contribute to somatic and germline cell tissues of chimaeric animals such as mice when re-introduced into the embryonic environment;

(c) it demonstrates selective binding to high affinity receptors on murine ES (EKcs-1 (previously known as CS1) and D3) and EC (PCC3-3A and F9) cells; and

(d) specific binding of ^{125}I-LIF to high affinity receptors is not in competition with insulin, IGF-I, IGF-II, acidic and basic FGF, TGFβ, TNFα, TNFβ, NGF, PDGF, EGF, IL-1, IL-2, IL-4, GM-CSF, G-CSF, Multi-CSF nor erythropoietin, but is in competition with murine and human LIF.

Accordingly, a first aspect of the present invention relates to a method for the isolation of embryonic stem (ES) cells from animal embryos in vitro which method comprises deriving ES cells from said embryos in culture medium, said culture medium containing an effective amount of leukaemia inhibitory factor (LIF), for a time and under conditions sufficient for the development of said ES cells. The embryos used may be isolated from animals including, but not limited to, humans and a number of other animal species such as birds (e.g. chickens), mice, sheep, pigs, cattle, goats and fish.

A second aspect of the present invention, contemplates a process for maintaining animal embryonic stem (ES) cells in vitro while retaining their pluripotential phenotype, which process comprises culturing said cells in a culture medium containing an effective amount of leukaemia inhibitory factor (LIF) under conditions sufficient to maintain said cells. The ES cells in accordance with this aspect of the invention include cells from humans, mice, birds (e.g. chickens), sheep, pigs, cattle, goats and fish.

The LIF used in the culture medium is preferably recombinant LIF produced, by way of example, in accordance with the methods described in International Patent Application No. PCT/AU88/00093 In accordance with the present invention, it has been found that recombinant LIF and in particular recombinant human and murine LIF are effective substitutes for, or additives to, feeder layers or conditioned medium in maintaining ES cells in vitro. For the purposes of the present description recombinant LIF is produced in *E. coli* and yeast using the methods described in International Patent Application No. PCT/AU88/00093, however, it is within the scope of the present invention to include recombinant LIF produced in other hosts including mammalian and insect cells and to synthetic LIF.

In another aspect, the present invention extends to ES cells derived from animal embryos by passage in a culture medium containing LIF, to such ES cells hav-

ing additional genetic material inserted therein, and to chimaeric animals such as chimaeric mice or transgenic progeny of said animals generated by known techniques using ES cells which have been maintained in vitro in a LIF-containing culture medium.

Thus, the invention extends to the generation and maintenance of ES cells from humans, mice, birds (e.g. chickens), sheep, pigs, cattle, goats and fish and to the generation of transgenic chimaeric animals and their transgenic progeny using the ES cells isolated from animal species such as mice, birds (e.g. chickens), sheep, pigs, cattle, goats and fish. This invention also includes the use of LIF in culture media to modulate the survival and growth of human and other animal species such as cattle germ cells and embryonic cells, for example, for use in in vitro fertilisation and other procedures.

The present invention may also be described by reference to the following figures:

FIGS. 1A and 1B are graphical representations showing the effect on ES cells of different concentrations of LIF.

FIGS. 2A, 2B, 2C, 2D, 2E and 2F are representations showing ES cell morphology in the presence and absence of LIF.

FIG. 3 is a graphical (3A and 3C) and pictorial (3B) representation showing the binding of ^{125}I-LIF to ES cells (EKcs-1) and EC cells (F9 and PCC3-A).

The present invention is directed to a method for the isolation and maintenance of embryonic stem (ES) cells from animal embryos in vitro which method comprises deriving and/or maintaining said ES cells from said embryos in culture medium containing an effective amount of leukaemia inhibitory factor (LIF), for a time and under conditions sufficient for the derivation and/or maintenance of said ES cells. The animal embryos may be isolated from a number of animal species such as humans, mice, birds (e.g. chickens), sheep, pigs, cattle, goats and fish. By reference herein to "animal embryos" includes reference to "animal blastocyts. Furthermore, the present invention is exemplified using human LIF with murine ES cells (heterologous system) and murine LIF with murine ES cells (homologous system) This is done with the understanding that the present invention contemplates LIF from any animal species in heterologous or homologous systems with animal embryos from animal species such as humans, mice, birds (e.g. chickens), sheep, pigs, cattle, goats and fish. Although in certain circumstances, a heterologous system will work effectively, it may be preferable to use homologous systems. Given the teachings herein, it will be routine for the skilled technician to ascertain whether a homologous or heterologous system is required in order to isolate or maintain particular animal ES cells.

By "culture medium" is meant a suitable medium capable of supporting growth of ES cells. Examples of suitable culture media useful in practicing the present invention are Eagles medium or modifications or equivalents thereof such as Dulbecco's or Glasgows modified Eagle's medium with supplements such as 5%-30% (v/v) foetal calf serum and where necessary 0.01 to 1.0 mM β-mercaptoethanol but preferably about 0.1 mm β-mercaptoethanol. The culture medium may or may not contain feeder cells and LIF may be used to substitute for, or add to, said feeder cells. When required, LIF, or more particularly synthetic or recombinant LIF, is added to the medium at a concentration of about 100–1,000,000 units/ml and preferably about 100–100,000 units/ml and even more preferably 500–10,000 units/ml where 50 units are defined as the amount of LIF which in one milliliter induces a 50% reduction in clone formation by murine M1 myeloid cells. By "recombinant LIF" is meant the LIF prepared by genetic engineering means such as, for example, according to International Patent Application No. PCT/AU88/00093 where a number of hosts such as bacteria (e.g. *E. coli*) or yeast cells may be employed. In accordance with the present invention, the effective derivation time is from 1 day to 20 weeks and particularly from 1 to 8 weeks.

Another aspect of the present invention contemplates a process for maintaining animal ES cells in vitro while retaining their pluripotential phenotype which process comprises culturing said cells in a culture medium containing an effective amount of LIF under conditions sufficient to maintain said cells. The ES cells in accordance with this aspect of the invention include cells derived from humans, mice, birds (e.g. chickens), sheep, pigs, cattle, goats and fish. As with the isolation of ES cells from animal embryos, the LIF used in the aforementioned process is preferably recombinant LIF. The culture medium may or may not contain feeder cells.

In accordance with the present invention, "pluripotential cells" and "embryonic stem cells" are those which retain the developmental potential to differentiate into all somatic and germ cell lineages.

The ability of recombinant LIF to maintain the stem cell phenotype of ES cells is demonstrated by transferring ES cells D3 and HD5 into normal cell culture medium in the presence of varying concentrations of purified yeast-derived recombinant human LIF (rY-HLIF) or *E. coli*—derived recombinant mouse LIF (rE-MLIF). At concentrations of 1000–5000 units/ml of rY-HLIF or rE-MLIF more than 90% of the D3 and HD5 ES cells retained their stem cell phenotype. In contrast, the ES cells maintained in normal culture medium differentiated over a period of 3–6 days. The proportion of colonies having the stem cell phenotype was related to the concentration of LIF in the culture medium. In addition to maintaining established ES cell lines, six new ES cell lines (MBL-1,2,3,4,5 & 6) were isolated from blastocysts in the absence of feeder cells when the media was supplemented with 1000 units/ml rE-HLIF Long term maintenance of the ES cell lines D3, HD5 and MBL-1 to 6 in LIF for up to 22 passages (approximately 100 cell generations or 10 weeks) did not noticeably alter the growth characteristics of these ES cells or their dose dependency on LIF. The ability of these ES cells to differentiate into all somatic and germ cell linages was confirmed by reintroduction of D3 and MBL-1 cells into blastocysts. Approximately 50% of the progeny analysed contained tissues derived from the injected ES cells with levels of overt chimaerism as high as 90% in individual mice. To test for germline transmission of ES derived cells male chimaeras were mated to C57BL/6J mice. Three D3 and two MBL-1 C57BL/6J chimaeras gave rise to agouti progeny confirming that these ES cells can contribute to the formation of germ cells.

The present invention also relates to chimaeric animals generated by known techniques using the ES cells contemplated herein. These ES cells may be isolated from animal embryos and/or maintained in vitro according to the subject invention. Furthermore, genetically manipulated ES cells may be passaged in LIF and used to make chimaeric animals. For example, genetically manipulated ES cells containing a retrovirus vec-

tor (N-TK527; derived from pXT1; C. A. Boulter and E. F. Wagner, (1987) Nucl. Acids Res. 15:7194) encoding genes for neomycin resistance and c-src^{527} were propogated in the presence of LIF but in the absence of feeder cells for over 20 passages. These cells still retained the ability to differentiate as judged by the formation of normal chimaeras following introduction of these cells into preimplantation embryos by blastocyst injection.

Further details of the use of LIF in accordance with the present invention will be apparent from the following Examples.

EXAMPLE 1

This example sets out the steps used to maintain ES cells in vitro in LIF, and to generate chimaeric mice using ES cells so passaged.

Step 1: Propagation in vitro

The ES cells used were the D3 (Doetschman, T. C. et. al. (1985) J.Embryol.Exp.Morphol. 87: 27–45) the EKcs-1 (Previously known as CS1) (Wagner, E. F. et.al. (1985) Cold Spring Harbor Symp.Quant.Biol. 50:691–700) and the HD5 (C. Stewart, unpublished) ES cell lines isolated from 129 SV He blastocysts and the CBL63 (R. Kemler, unpublished) ES cells isolated from C57BL/6J blastocysts. Prior to culture in LIF, the D3 and CBL63 cells were maintained in Dulbecco modified Eagles medium with 15% (v/v) foetal calf serum on a feeder layer of primary embryo fibroblasts, and the EKcs-1 and HD5 ES cells were maintained in Eagle's medium with 15% (v/v) foetal calf serumand 0.1 mM β-mecraptcethanol, in the presence of medium conditioned by the bladder carcinoma cell line 5637 (ATCC No.HTB9).

The ability of recombinant LIF to maintain the stem cell phenotype of ES cells was demonstrated by transferring ES cells of the lines D3 and HD5 into normal cell culture medium in the presence of varying concentrations of purified yeast-derived recombinant human LIF (hereafter referred to as rY-HLIF), or *E.coli* derived recombinant mouse LIF (rE-MLIF) (previously disclosed in International Patent Application No. PCT/AU88/00093). The results are shown in FIGS. 1 and 2. In FIG. 1A, HDS cells previously maintained in 80% 5637 conditioned medium for eight passages were transferred to culture media containing 0–5,000 units ml^{-1} of purified, recombinant yeast-derived human LIF (H-LIF; see below) ( ) or purified, recombinant *E. coli*-derived mouse LIF (M-LIF; see below) (o—o). HD5 cells maintained in medium containing 1,000 units ml^{-1} H-LIF for a further 13 passages were then transferred to 0–1,000 units ml^{-1} M-LIF ( ). In FIG. 1B, D3 cells maintined on mouse embryo fibroblasts for 10 passages were transferred to media containing 1,000–5,000 units ml^{-1} H-LIF and after a further 7 or 15 passages the cells were transferred into media containing 0–5,000 units ml^{-1} of H-LIF ( ) or 0–1,000 units m^{-1} M-LIF ( ) respectively. FIG. 2 shows ES cell morphology in the presence of recombinant LIF. HD5 ES cells cultured in the presence of 80% 5637 conditioned medium were assayed for the ability of purified recombinant LIF to maintain the stem-cell phenotype by transfer to media containing 1 000 units ml^{-1} M-LIF (A), or to normal culture media (B). After seven days, the colonies were srained with Giemsa. Compact stem-cell colonies could be distinguished from diffuse differentiated colonies. D3 cells maintained in H-LIF for 15 passages were assayed for the ability to differentiate by transfer into media containing 1,000 units ml^{-1} M-LIF (C) or normal culture media (D). Immunofluorescence of the cells in the two D3 colony types was carried out using the ECMA-7 monoclonal antibody which recognizes a stem cell-specific cell-surface antigen. Cell-surface-specific immunofluorescence was detected on over 90% of the cells maintained in media containing 1,000 units ml^{-1} recombinant LIF (E) but on less than 1% of the cells maintained in normal culture media (F). The field of view shown in (F) contains 21 cells.

FIGS. 1 and 2 indicate that over 90% of the ES cells maintained in 1000–5000 units/ml rY-HLIF or rE-MLIF retained their stem cell phenotype. In contrast, ES cells maintained in normal culture medium differentiated over a period of 3–6 days. The different concentrations of rY-HLIF or rE-MLIF used did not result in any noticeable change in cell number after 6 days in culture, indicating that there is no selection for a specific subpopulation able to grow in LIF. Similar results have been obtained using yeast-derived rMLIF also disclosed in International Patent Application No. PCT/AU88/00093. The data in FIG. 1 indicate that human LIF acts on mouse ES cells, as previously described for the action of human LIF on M1 myeloid leukaemic cells (Gough, N. M. et. al. (1988) Proc.Natl.Acad.Sci.USA 85: 2623–2627). The data in FIG. 1 also indicate that the action of LIF on ES cells is independent of glycosylation, as previously described for the action of LIF on M1 myeloid leukaemic cells.

Four ES cell lines, D3, EKcs-1, CBL63 and HD5, were maintained in medium containing 1000–5000 u/ml rY-HLIF for up to 22 passages (10 weeks or approximately 100 generations). Long-term maintenance of the ES cells in rY-HLIF did not noticeably alter the growth characteristics of the cells. Furthermore, reduction or removal of the LIF from the culture medium resulted in the differentiation of the ES cells with similar kinetics to those explanted directly from bladder carcinoma 5637 conditioned ..edium or a feeder layer of mouse fibroblasts (for example, see FIGS. 1 and 2). The stem cell phenotype of ES cells cultured for multiple passages in the presence of LIF was confirmed by immunofluorescence with the ECMA-7 antibody which recognises a cell-surface stem-cell-specific antigen (Kemler, R. in Progress in Developmental Biology Band 26 Sauer, H. W. ed page 175; Fisher, Stuttgart, 1980); ES cells cultured in the presence of LIF expressed the stem cell marker, whereas in the absence of LIF less than 1% did so (FIG. 2).

Step 2: Isolation of ES cell lines

Murine blastocysts were isolated from 129 Sv He mice at day 4 of development (day 1 = day of plug) into either Dulbecco's or Glasgows modified Eagle's medium with 15% (v/v) foetal calf serum, 0.1 mM β-mercaptoethanol and 1000 units/ml of purified rE-HLIF. ES cell lines were then isolated by two different methodologies.

In the first method the blastocysts were allowed to attach to the culture dish and approximately 7 days later the outgrowing inner cell mass picked, trypsinised and transfered to another culture dish in the same culture media. ES cell colonies appeared 2–3 weeks later with between 5–7 individual colonies arising from each explanted inner cell mass. The ES cell lines were then expanded for further analysis. The second method for isolation of ES cell lines used the immunosurgery technique (described in Martin, G. R. (1981) Proc. Natl.

Acad. Sci. USA 78:7634–7638) where the trophectoderm cells are destroyed using anti-mouse antibodies prior to explanting the inner cell mass. The efficiency of ES cell lines isolation is shown in Table 1.

Step 3: Generation of Chimaeric Mice

All the ES cell lines cultured in the absence of feeder cells but in the presence of LIF (referred to in step 1) or directly isolated with the aid of culture medium containing LIF (referred to in step 2) retained the ability to differentiate into multiple cell types following the removal of LIF indicating that these cells have retained their pluripotential phenotype. To confirm their developmental potential, D3 ES cells maintained in LIF for 7–22 passages and MBL-1 ES cells maintained in LIF for 14–17 passages were reintroduced into the embryonic environment by blastocyst injection (as described in Williams et al., (1988) Cell 52:121–131). Blastocysts were isolated from the outbred ICR mouse strain or inbred C57BL/6J mice. The expanded blastocysts were maintained in oil-drop cultures at 4° C. for 10 min prior to culture. The ES cells were prepared by picking individual colonies, which were then incubated in phosphate-buffered saline, 0.5 mM EGTA for 5 min; a single cell suspension was prepared by incubation in a trypsin-EDTA solution containing 1% (v/v) chick serum for a further 5 min at 4° C. Five to twenty ES cells (in Dulbecco's modified Eagle's Medium with 10% (v/v) foetal calf serum and 3,000 units/ml DNAase 1 buffered in 20 mM HEPES [pH 8]) were injected into each blastocyst. Blastocysts were transferred into pseudopregnant recipients and allowed to develop normally. Chimaeric mice were identified by coat markers (Hogan et al., (1986) Manipulating the Mouse Embryo, Cold Spring Harbor, N.Y.). Analysis of the subsequent chimaeric mice revealed that up to approximately 50% of the progeny contained tissues derived from the injected cells (Table 2), with levels of overt chimaerism as high as 90% in individual mice. Furthermore analysis of the organs of four D3-chimaeras confirmed that the ES cells maintained in LIF could contribute extensively to the development of all of the somatic tissues (Table 3).

The male chimaeras were tested for germline transmission of ES derived cells by mating to ICR or C57BL/6J females. Three out of four of the D3-C57BL/6J chimaeras and two out of six of the MBL-1-C57BL/6J chimaeras gave rise to agouti offspring derived from the ES cells cultured in LIF (Table 4).

To test whether genetically altered ES cells could be maintained in culture medium containing LIF, D3 ES cells were infected with a retrovirus vector (N-TK527) expressing the neomycin resistance gene and a C-src gene mutant (c-src^{527}) (protocol for infection is described in Williams et al., (1988) Cell 52: 121–131). The ES cell clones isolated were maintained in culture medium containing LIF for over 20 passages. These genetically modified ES cells retained the ability to form chimaeric mice following reintroduction into the embryonic environment by blastocyst injection (Table 2)

TABLE 1

Isolation of 129 Sv He ES cell lines in media containing rE-HLIF

Methodology	Blastocyst	ICM outgrowing	Number of ES cell lines derived
Explanted	9	9	4
Immunosurgery	11	3	0

TABLE 1-continued

Isolation of 129 Sv He ES cell lines in media containing rE-HLIF

Methodology	Blastocyst	ICM outgrowing	Number of ES cell lines derived
Immunosurgery	7	5	2

Murine blastocysts were isolated from 129 Sv He mice at day 4 of development (day 1=day of plug) into either Dulbecco's or Glasgows modified Eagle's medium with 15% (v/v) foetal calf serum, 0.1 mM β-mercaptoethanol and 1000 units/ml of purified rE-HLIF. The blastocysts were then explanted into the same media and left to attach to the culture dish and the inner cell mass picked dissociated in phosphate-buffered saline, 0.5 mM EGTA for 5 min; a single cell suspension was prepared by incubation in a trypsin-EDTA solution containing 1% (v/v) chick serum and the cells replated in the cell culture medium described above. The characteristic ES cell colonies appeared within 1–3 weeks.

Other blastocysts were treated by immunosurgery (as described in Martin, G. R. (1981) Proc. Natl. Acad. Sci. USA 78:7634–7638). The blastocysts were allowed to hatch from the zona pelucida, and then treated with anti-mouse antibodies and destroyed by the addition of complement. The exposed inner cell mass was then left to attach to a tissue culture dish and again treated with anti-mouse antibodies and complement. Within a few days pluripotential stem cell colonies appeared and were dissociated and trypsinised as described above.

TABLE 2

Chimaeric mice derived from ES cells cultured in LIF

ES cells	Blastocysts transferred	Pups born	Chimaeras
D3	142	60 (42%)	33 (55%)
MBL-1	51	33 (65%)	16 (48%)
D3 N-TK527	42	22 (52%)	12 (54%)

TABLE 3

Percentage tissue contributions in individual D3 chimaeric mice

Chimaera	Necropsy age	C	Bl	Sp	P	Ll	T	H
D3-1	13d	35	0	35	20	10	20	40
D3-2	14d	40	15	35	30	45	30	50
D3-3	11d	90	50	50	35	50	40	60
D3-4	11d	50	50	50	30	40	40	50
		Lu	G	K	M	B	Sa	
D3-1	13d	30	10	35	30	35	20	
D3-2	14d	35	20	30	50	50	25	
D3-3	11d	45	50	50	70	50	55	
D3-4	11d	50	35	50	50	20	30	

TABLE 4

Chimaeric demonstrating germline transmission of ES derived cells.

Mice	Chimaerism	Passage no. of D3 cells on feeders	Passage no. of D3 cells in LIF	Offspring 129 Sv He	Offspring C57
775-3	75%	10	16	9	24
778-1	70%	10	22	5	33
778-2	90%	10	22	2	36
778-3	55%	10	22	0	0

The following relates to Tables 2, 3 and 4:

D3 and MB1-1 ES cells are derived from 129 Sv He mice (inbred, agouti, homozygous for the glucose phosphate isomerase 1^a allele). The D3 ES cells were origi-

nally cultured on primary embryo fibroblasts for 10 passages and then transferred to 1,000–5,000 units/ml recombinant LIF for 7–22 passages. The MB1-1 ES cells were isolated in the absence of feeder cells but in the presence of rE-HLIF these cells were cultured for 14–17 passages. The ES cells were then injected into ICR (outbred, albino) or C57BL/6J (inbred, black) blastocysts which were then transferred into pseudo-pre.gnant foster mothers. Both the ICR and C57BL/6J mice are homozygous for the glucose phosphate isomerase 1^b allele. Chimaeric pups were identified by coat pigmentation (only foster mothers which became pre.gnant were counted in estimating the number of progeny). Tissue chimaerism was estimated using glucose phosphate isomerase strain differences. The extent of tissue chimaerism was determined in two D3-ICR (numbers 1 and 2) and two D3-C57BL/6J chimaeras (numbers 3 and 4). Tissues analysed: C, coat; Bl, blood; Sp, spleen; P, pancrease; Li, liver; T, thymus; H, heart; Lu, lungs; G, gonads; K, kidneys; M, muscle; B, brain; Sa, salivary gland. Male chimaeras were mated to ICR or C57BL/6J mice and offspring identified by coat pigmentation.

EXAMPLE 2

This example sets out the steps used to document specific high affinity receptors on ES and EC cells. Accompanying FIG. 3 shows binding of ^{125}I-LIF to ES cells EKcs-1 and EC cells F9 and PCC3-A (Jakob, J. et. al. (1973) Ann.Microbiol.Inst.Pasteur, 124B:. 269–282). In relation to FIG. 3, (A), Scatchard analysis of ^{125}I-labelled LIF binding to F9 ( ), EKcs-1 ( ), PCC3A-1 ( ) and M1 (o) cells. Saturation curves for binding were analysed by the method of Scatchard by plotting the amount of LIF specifically bound (defined as the difference between binding observed in the absence and presence of excess unlabelled LIF) versus the ratio of bound to free LIF. Free LIF values were adjusted for the percent of ^{125}I-labelled LIF capable of binding specifically to LIF receptors, in this experiment determined to be 75%. The apparent dissociation constant for the interaction of LIF with its receptor was determined from the slopes of the curves and the receptor number from their intercepts with the ordinate. Results in (A) were standardized to 5×10^6 cells per point and the mean of duplicate points are shown and curves were fitted using the Ligand program (B), Autoradiography of F9 EC cells labelled with ^{125}I-labelled LIF. (C), Quantitation of silver grains on F9 EC cells after binding of ^{125}I-labelled LIF.

Purified recombinant (yeast-derived) human LIF (rY-HLIF) was radioactively labelled on tyrosine residues as described previously (Hilton, D. J. et.al. (1988) Proc.Natl.Acad.Sci. USA., 85:5971–5975) producing ^{125}I-LIF with a specific radioactivity of approximately 1.2×10^7 cpm/pmole. ^{125}I-LIF (2×10^3–5×10^5 cpm) was incubated with $1–4\times10^6$ target cells with or without at least 100-fold molar excess of unlabelled LIF, in a total volume of 100 μl for 4 hours on ice. Cell-associated and free ^{125}I-LIF were separated by centrifugation through foetal claf serum (Nicola, N. A. and Metcalf, (1986) D. J.Cell Physiol. 128:160–188). Specific cell-associated ^{125}I-LIF was determined by cold competition.

FIG. 3 illustrates the specific saturable and high affinity binding of ^{125}I-LIF to the ES cells EKcs-1 and the EC cells PCC3-A and F9. The number of LIF receptors per cell derived from these Scatchard plots were 295, 190 and 330, respectively, with apparent dissociation constants at 4° C. of approximately 90 pM. This compares with the M1 cell line, a LIF-responsive monocytic leukaemia, which displays 50–200 LIF receptors/cell with an apparent dissociation constant of 50–150 pM. All other ES and EC cells tested - D3, NG2, PC13 and P19 - bound similar levels of LIF (data not shown).

The binding of ^{125}I-LIF to M1 cells, EKcs-1 and PCC3-A was also found to be in competition with unlabelled recombinant and native murine and human LIF, but not with the range of other hormones and factors, (including several which act on embryonic cells): insulin, IGF-I, IGF-II, acidic and basic FGF, TGFβ, TNFα, TNFβ, NGF, PDGR, EGF, IL-1, IL-4, GM-CSF, G-CSF, Multi-CSF and erythropoietin.

We claim:

1. A method for the isolation of embryonic stem (ES) cells from mammalian embryos in vitro which method comprises deriving and maintaining said embryos in culture medium containing an effective amount of recombinant leukaemia inhibitory factor (LIF) for a time and under conditions sufficient for the development of said ES cells.

2. The method according to claim 1 wherein the culture medium is free of feeder cells.

3. The method according to claim 1 wherein the culture medium is Eagle's medium or modifications thereof or equivalent thereto.

4. The method according to claim 1 wherein the LIF is recombinant human or murine LIF.

5. The method according to claim 4 wherein LIF is added to the culture medium at a concentration of from 10 to 1,000,000 units/ml.

6. The method according to claim 5 wherein the LIF is added to the culture medium at a concentration of from 100 to 100,000 units/ml.

7. The method according to claim 6 wherein the LIF is added to the culture medium at a concentration of from 500 to 10,000 units/ml.

8. A method according to claim 1 wherein the effective time is from 1 day to 20 weeks.

9. The method according to claim 8 wherein the effective time is from 1 to 8 weeks.

10. A method for maintaining mammalian embryonic stem (ES) cells in vitro while retaining their pluripotential phenotype which process comprises culturing said cells in a culture medium containing an effective amount of recombinant leukaemia inhibitory factor (LIF) under conditions sufficient to maintain said cells.

11. The method according to claim 10 wherein the culture medium is free of feeder cells.

12. The method according to claim 10 wherein the culture medium comprises Eagle's medium or modifications thereof or equivalents thereto.

13. The method according to claim 10 wherein the LIF is recombinant murine or human LIF.

14. The method according to claim 13 wherein the recombinant LIF is added to the culture medium at a concentration of from 10 to 1,000,000 units/ml.

15. The method according to claim 14 wherein the recombinant LIF is added to the culture medium at a concentration of from 100 to 100,000 units/ml.

16. The method according to claim 15 wherein LIF is added to the culture medium at a concentration of from 500 to 10,000 units/ml.

* * * * *

US005453357A

United States Patent [19]

Hogan

[11] **Patent Number: 5,453,357**

[45] **Date of Patent: Sep. 26, 1995**

[54] **PLURIPOTENTIAL EMBRYONIC STEM CELLS AND METHODS OF MAKING SAME**

[75] Inventor: **Brigid L. M. Hogan**, Brentwood, Tenn.

[73] Assignee: **Vanderbilt University**, Nashville, Tenn.

[21] Appl. No.: **958,562**

[22] Filed: **Oct. 8, 1992**

[51] **Int. Cl.**[6] **C12N 5/06**; C12Q 1/02

[52] **U.S. Cl.** **435/7.21**; 435/21; 435/29; 435/34; 435/240.2; 435/240.21; 435/240.3

[58] **Field of Search** 435/7.21, 29, 240.2, 435/240.21, 240.3, 21, 34

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Primary Examiner—David Saunders
Attorney, Agent, or Firm—Needle & Rosenberg

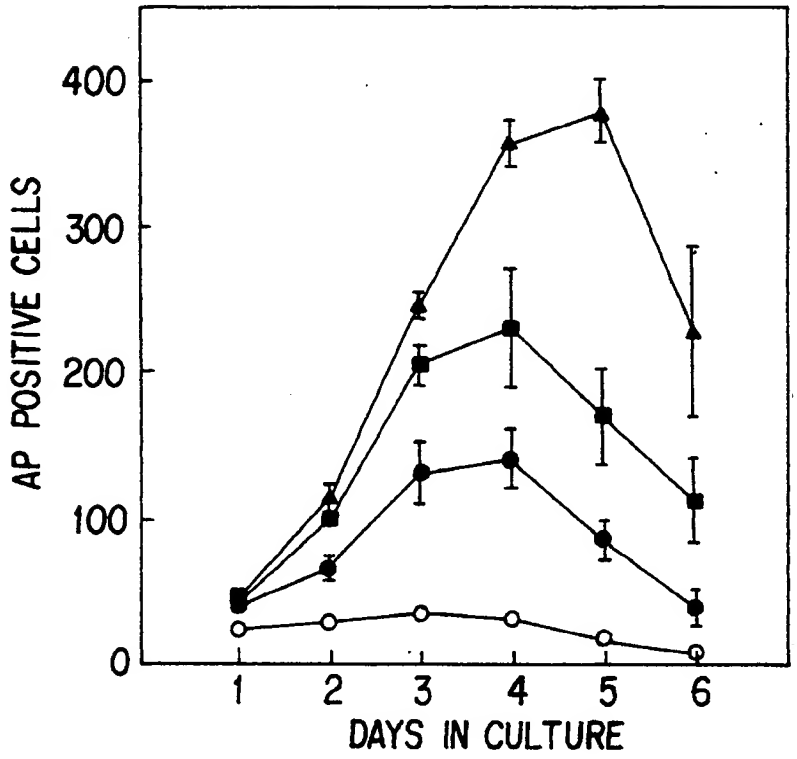
[57] **ABSTRACT**

The present invention provides a non-mouse pluripotential embryonic stem cell which can:

(a) be maintained on feeder layers for at least 20 passages; and

(b) give rise to embryoid bodies and multiple differentiated cell phenotypes in monolayer culture. The invention further provides a method of making a pluripotential embryonic stem cell comprising administering a growth enhancing amount of basic fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor to primordial germ cells under cell growth conditions, thereby making a pluripotential embryonic stem cell.

14 Claims, 5 Drawing Sheets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4
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DAYS IN CULTURE

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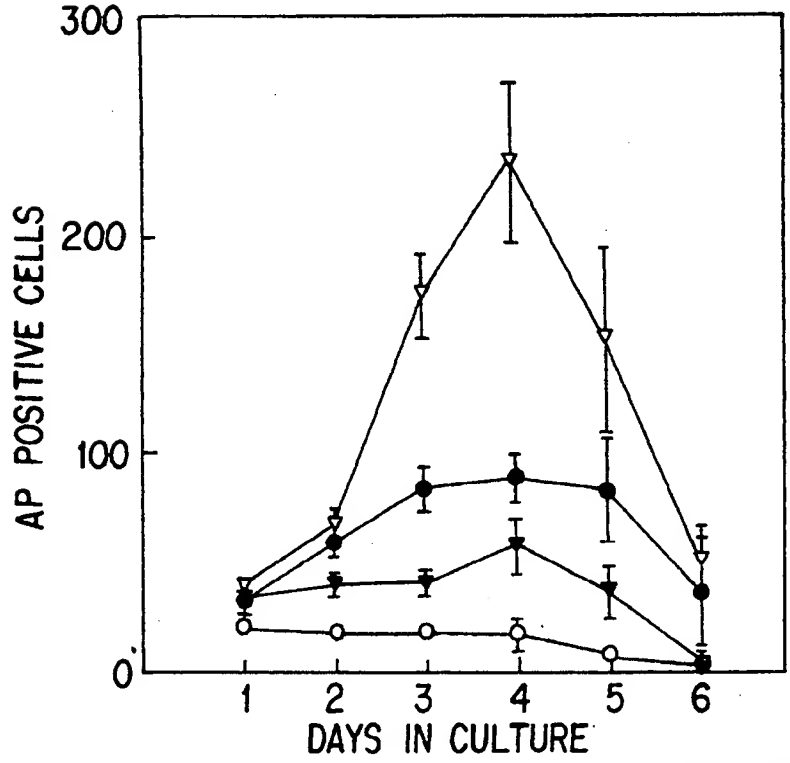
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DAYS IN CULTURE

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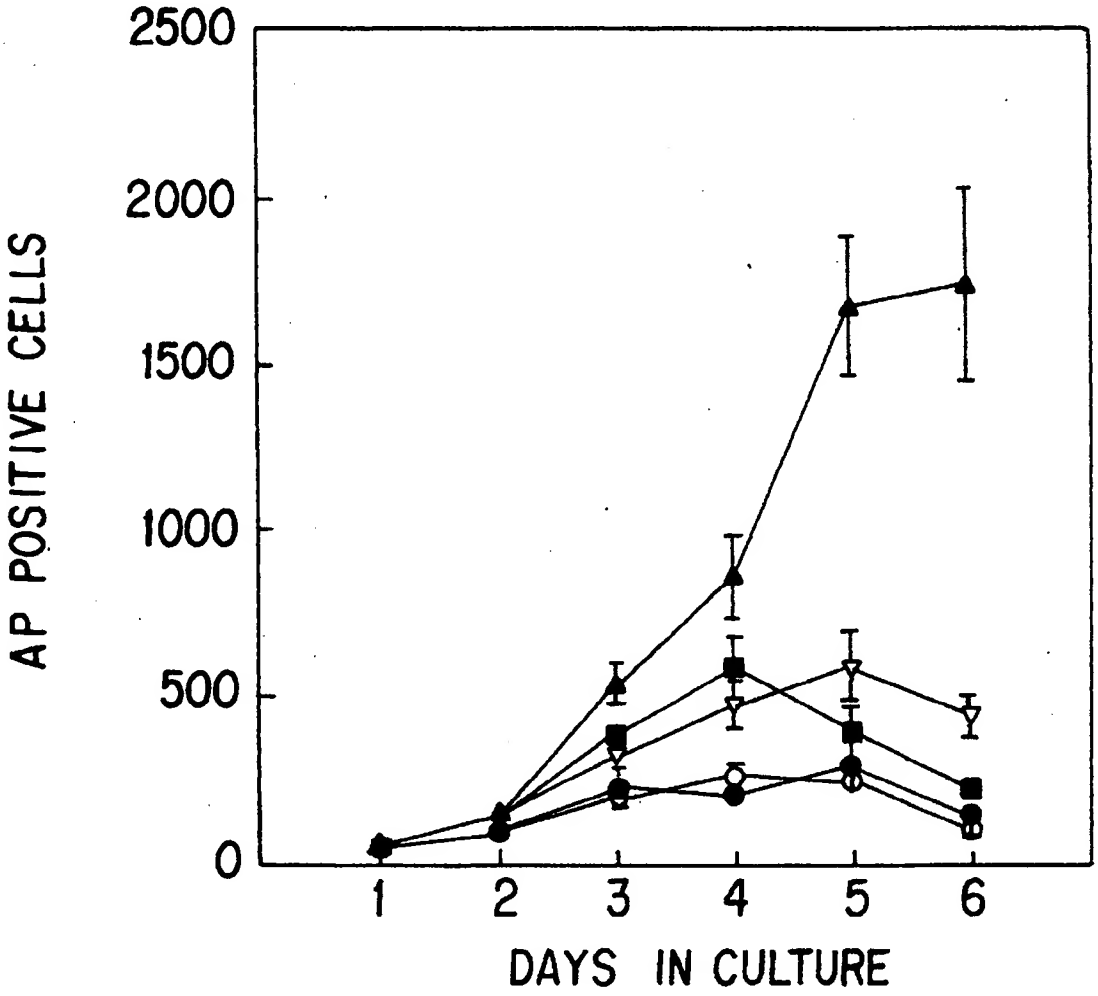
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AP POSITIVE CELLS
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2
3
4
5
6
DAYS IN CULTURE

FIG. 1C

FIG.2C

FIG.2F

FIG.2I

FIG.2B

FIG.2E

FIG.2H

FIG.2A

FIG.2D

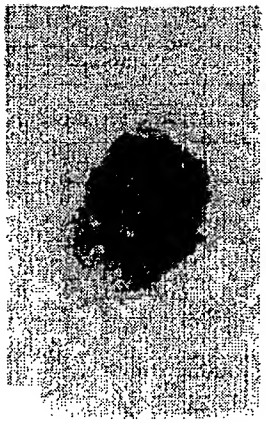
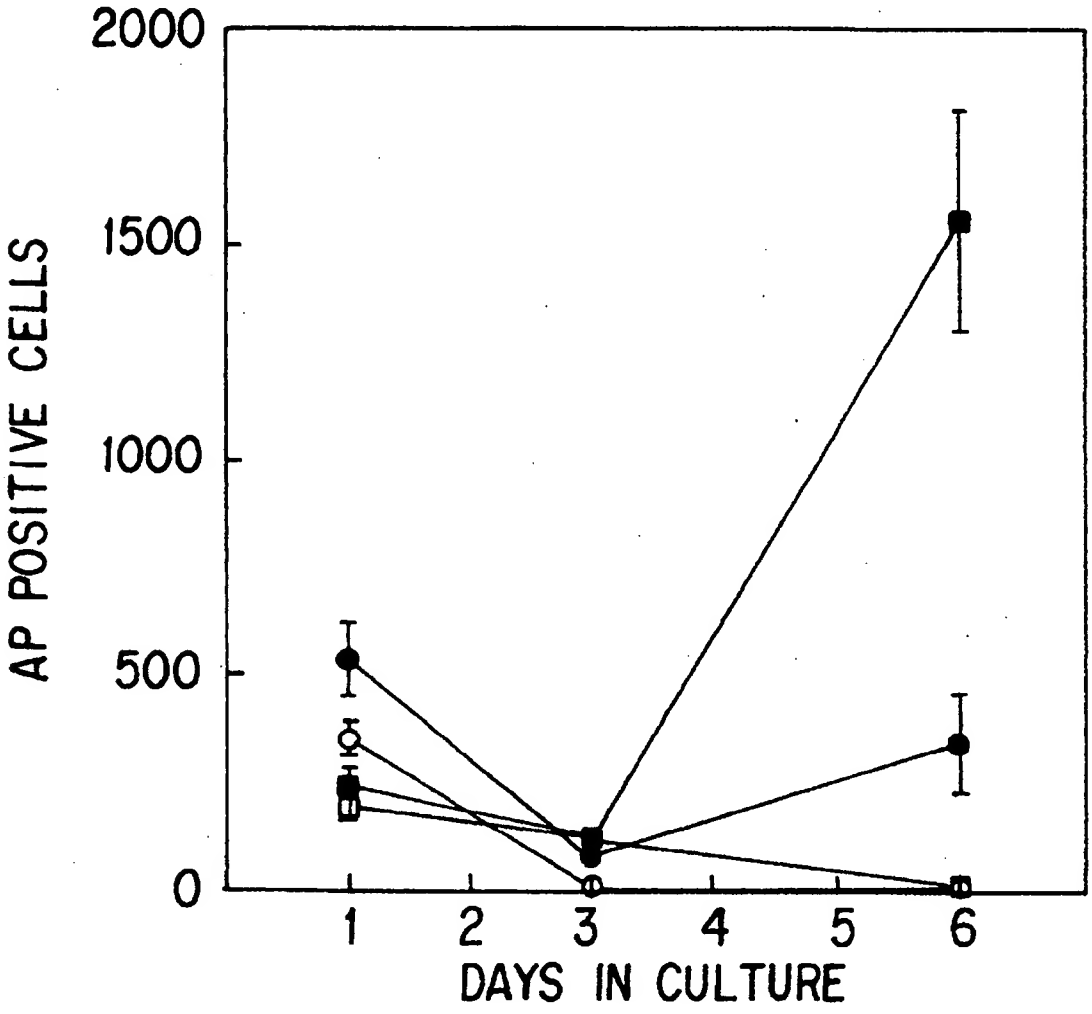


FIG.2G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DAYS IN CULTURE

FIG. 3

FIG.4B

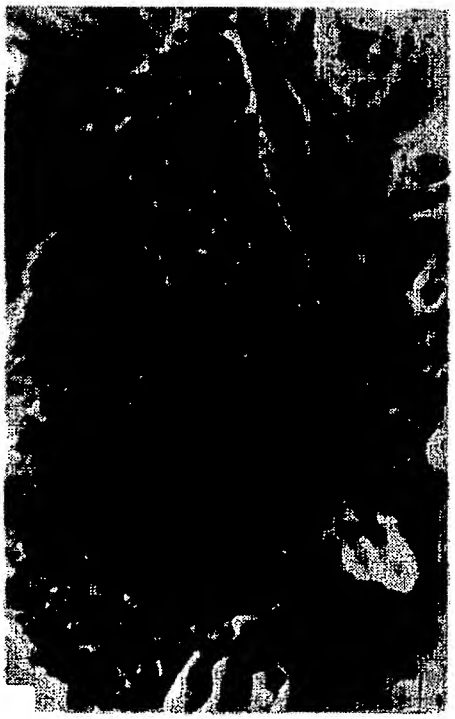

FIG.4A

FIG.4E

FIG.4D

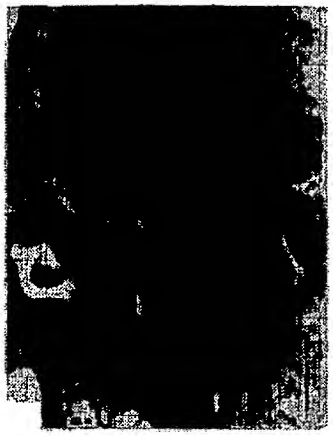

FIG.4C

PLURIPOTENTIAL EMBRYONIC STEM CELLS AND METHODS OF MAKING SAME

This invention was made with government support under grant number HD25580-04 from the National Institute of Health Child Health and Development. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to pluripotential embryonic stem cells and methods and compositions for making pluripotential embryonic stem cells.

2. Background Art

Primordial germ cells (PGCs) in the mouse are thought to be derived from a small population of embryonic ectoderm (epiblast) cells set aside at the egg cylinder stage prior to gastrulation (Lawson and Pederson, 1992), or even earlier (Soriano and Jaenisch, 1986). By 7 days post coitum (p.c.) about 100 alkaline phosphatase (AP) positive PGCs can be detected in the extra embryonic mesoderm just posterior to the definitive primitive streak (Ginsberg et al., 1990). These cells continue to proliferate and their number increases rapidly to around 25,000 at 13.5 days p.c. (Mintz and Russell, 1957; Tam and Snow, 1981). At the same time the PGCs migrate from the base of the allantois along the hind gut and reach the genital ridges by 11.5 days p.c. In the genital ridge, PGCs stop dividing at around 13.5 days p.c., and enter either mitotic arrest in the developing testis or meiosis in the ovary. In a few strains of mice, e.g. 129, this normal program can be disrupted if the male genital ridge from an 11.5 to 12.5 days p.c. embryo is grafted to an ectopic site such as the testis or kidney capsule. Under these conditions some PGCs give rise to teratomas and transplantable teratocarcinomas containing pluripotential embryonal-carcinoma (EC) stem cells (Stevens and Makensen, 1961; Stevens, 1983; Noguchi and Stevens, 1982).

Previous studies have shown that steel factor (SF) and leukemia inhibitory factor (LIF) synergistically promote the survival and in some cases the proliferation of mouse PGCs in culture (Godin et al., 1991; Dolci et al., 1991; Matsui et al., 1991). However, under these conditions, PGCs have a finite proliferative capacity that correlates with their cessation of division in vivo. A similar finite proliferative capacity has been reported for oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells in the rat optic nerve. In this case, PDGF is involved in the self renewal growth of O-2A cells (Noble et al., 1988; Raff et al., 1988). After a determined number of cell divisions, O-2A cells may lose their responsiveness to PDGF and start differentiating into oligodendrocytes. If both PDGF and basic fibroblast growth factor (bFGF) are added in culture, O-2A progenitor cells keep growing without differentiation (Bogler et al., 1990).

Since pluripotential embryonic stem cells (ES) can give rise to virtually any mature cell type they are of great value for uses such as creating genetically manipulated animals. However, it has previously been possible only to obtain ES cells from mice. These mice ES cells were obtained from cultures of early blastocytes. Attempts at isolating ES cells from other animals have failed. Therefore, there is a great need to produce and maintain ES cells from a variety of different animals.

The present invention satisfies this need by demonstrating that, in the presence of bFGF, SF and LIF, PGCs continue to proliferate in culture and give rise to colonies of ES cells.

These stem cells can give rise to a wide variety of mature, differentiated cell types both in vitro and when injected into nude mice and when combined with embryos to form a chimera.

SUMMARY OF THE INVENTION

The present invention provides a non-mouse pluripotential embryonic stem cell which can:

(a) be maintained on feeder layers for at least 20 passages; and

(b) give rise to embryoid bodies and multiple differentiated cell phenotypes in monolayer culture. The invention further provides a method of making a pluripotential embryonic stem cell comprising administering a growth enhancing amount of basic fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor to primordial germ cells under cell growth conditions, thereby making a pluripotential embryonic stem cell.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the effect of growth factors on murine PGCs in culture.

(A) PGCs from 8.5 day p.pc. embryos were seeded into wells containing Sl/Sl^4 feeder cells either alone (open circles) or with soluble rSF (closed circles), soluble rSF and LIF (closed squares), or soluble rSF, LIF and bFGF (closed triangles). Cultures were fixed and the number of AP positive cells counted.

(B) As in (A) except that cells were cultured without added factors (open circles), with soluble rSF (closed circles), with bFGF (closed triangles) or with soluble rSF and bFGF (open triangles).

(C) As in (A) except that cells were cultured on Sl^4-m220 cells either alone (open circles) or with soluble rSF (closed circles), soluble rSF and LIF (closed squares), soluble rSF and bFGF (open triangles) and soluble rSF, LIF and bFGF (closed triangles).

Each experiment was carried out with duplicate wells and numbers are the means +s.e.m. of three separate experiments.

FIG. 2 shows the morphology of primary and secondary cultures of PGCs and their descendants. PGCs from 8.5 d p.c. embryos (A-E, G,H) or 12.5 d p.c. male genital ridges (F) were cultured on Sl^4-m220 cells as described and stained for AP activity.

(A) Primary culture after 4 days in the presence of LIF. Note that the AP positive cells are scattered among the feeder cells.

(B) Primary culture after 4 days in the presence of soluble rSF, LIF and bFGF. Note that the AP positive cells now form tight clumps.

(C) As for B, but after 6 days in culture.

(D) Secondary culture after 6 days in the presence of soluble rSF, LIF, and bFGF In this colony all the cells are AP positive.

(E) As for D except that cells at the edges of the colony are AP negative.

(F) PGCs from 12.5 day p.c. male genital ridge were cultured for 6 days in the presence of soluble rSF, LIF and bFGF. Colonies of tightly packed AP positive cells are present.

(G) Colony of ES-like cells in a secondary culture with

soluble SF, LIF and bFGF stained with SSEA-1 monoclonal antibody and for AP activity. Phase contrast microscopy.

(H) The same colony as in G viewed by fluorescence microscopy. AP positive cells also express SSEA-1.

(I) Colony grown under same conditions as (G) but stained without primary antibody Scale bars=200 um.

FIG. 3 shows the effect of growth factors on male and female PGCs in culture. Cells were dissociated from either male (squares) or female (circles) genital ridges from 12.5 day p.c. mouse embryos and cultured on Sl^4-m220 feeder cells either alone (empty symbols) or with soluble rSF, LIF and bFGF (filled symbols). Cells were fixed and the number of AP positive cells counted. The experiment was carried out three times, with duplicate wells.

FIG. 4 shows the morphology of undifferentiated PGC derived ES cells and their differentiated derivatives.

(A) Colony of densely packed ES-like cells obtained from PGCs of an 8.5 day p.c. embryo grown on Sl^4-m220 cells in the presence of soluble rSF, LIF and bFGF for 6 days. Scale bar=100 um.

(B) Simple embryoid bodies with an outer layer of endoderm (arrows) obtained after culturing PGC-derived ES cells for 4 days in suspension.

(C) Section of a teratoma obtained by injecting ES-like cells derived from PGCs of an 8.5 day p.c. embryo into a nude mouse. The region shown here contains neural tissue and pigmented epithelium. Scale bar=200 um.

(D) Region of the same tumor as in (C) showing a dermoid cyst and secretory epithelium.

(E) Region of the same tumor as in C and D, showing bone and cartilage. The differentiated tissues shown in C-E were seen in addition to other tissue types in multiple tumors from all three lines tested.

DETAILED DESCRIPTION OF THE INVENTION

The term "embryonic ectoderm" is used herein. "Embryonic ectoderm" and "epiblast" can be used interchangeably to refer to the same cell type.

A "pluripotential embryonic stem cell" as used herein means a cell which can give rise to many differentiated cell types in an embryo or adult, including the germ cells (sperm and eggs). This cell type is also referred to as an "ES cell" herein.

A "fibroblast growth factor" (FGF) as used herein means any suitable FGF. There are presently seven known FGFs (Yamaguchi et al. (1992)). These FGFs include FGF-1 (acidic fibroblast growth factor), FGF-2 (basic fibroblast growth factor), FGF-3 (int-2), FGF-4 (hst/K-FGF), FGF-5, FGF-6, and FGF-7. Each of the suitable factors can be utilized directly in the methods taught herein to produce or maintain ES cells. Each FGF can be screened in the methods described herein to determine if the FGF is suitable to enhance the growth of or allow continued proliferation of ES cells or their progenitors.

"Steel factor" (SF) is used herein. SF is also called stem cell factor, mast cell growth factor and c-kit ligand in the art. SF is a transmembrane protein with a cytoplasmic domain and an extracellular domain. Soluble SF refers to a fragment cleaved from the extracellular domain at a specific proteolytic cleavage site. Membrane associated SF refers to both normal SF before it has been cleaved or the SF which has been altered so that proteolytic cleavage cannot take place.

This invention provides a non-mouse pluripotential ES cell which can be maintained on feeder layers for at least 20 passages, and give rise to embryoid bodies and multiple differentiated cell phenotypes in monolayer culture. Only those non-mouse animals which can be induced to form ES cells by the described methods are within the scope of the invention. Given the methods described herein, an ES cell can be made for any animal. However, mammals are preferred since many beneficial uses of mammalian ES cells exist. Mammalian ES cells such as rats, rabbits, guinea pigs, goats, pigs, cows, and humans can all be obtained. Alternatively, embryos from these animals can be screened for the ability to produce ES cells. While the ES cells are non-mouse, it is possible that the ES cells produced by the combination of FGF, LIF and SF physically differ from the existing established murine ES cells. Thus, murine ES cells produced by the addition of FGF, LIF and SF are also contemplated.

The ES cells of this invention can be maintained for at least 20 passages. However, the ES cells may be capable of indefinite maintenance.

Once the non-mouse ES cells are established, they can be genetically manipulated to produce a desired characteristic. For example, the ES cells can be mutated to render a gene non-functional, e.g. the gene associated with cystic fibrosis or an oncogene. Alternatively, functional genes can be inserted to allow for the production of that gene product in an animal, e.g. growth hormones or valuable proteins. Such methods are very well established in the art (Sedivy and Joyner (1992)).

The invention also provides a composition comprising:

(a) pluripotential ES cells and/or primordial germ cells and/or embryonic ectoderm cells; and

(b) an FGF, LIF, membrane associated SF, and soluble SF in amounts to enhance the growth of and allow the continued proliferation of the cell.

Also provided is a composition comprising an FGF, LIF, membrane associated SF, and soluble SF in amounts to enhance the growth of and allow the continued proliferation of embryonic ectoderm or primordial germ cells.

The compositions arise from the fact that FGF, LIF and SF are used either to enhance the growth and proliferation of primordial germ cells or embryonic ectoderm cells to become ES cells. Growth and proliferation enhancing amounts can vary. Generally, 0.5 to 500 ng/ml of culture solution is adequate. Preferably, the amount is between 10 to 20 ng/ml.

Alternatively, FGF, LIF, and SF can be used to maintain ES cells. The amounts of FGF, LIF and SF necessary to maintain ES cells can be much less than that required to enhance growth or proliferation to become ES cells. In addition, FGF, LIF or SF may not be required for maintenance of ES cells.

In general, FGF or LIF from a species different from the source of the ES, primordial germ cell or embryonic ectoderm can be utilized. In addition, the SF utilized is preferably from the same species as the utilized cell type. However, FGF, LIF or SF from various species can be routinely screened and selected for efficacy in a cell from a different species.

The invention also provides a method of making a pluripotential ES cell comprising administering a growth enhancing amount of basic FGF, LIF, membrane associated SF, and

soluble SF to primordial germ cells and/or embryonic ectoderm cells under cell growth conditions, thereby making a pluripotential ES cell. This method can be practiced utilizing any animal cell, especially mammal cells including mice, rats, rabbits, guinea pigs, goats, cows, pigs, humans, etc. The ES cell produced by this method is also contemplated. "Cell growth conditions" are set forth in the Examples. However, many alterations to these conditions can be made and are routine in-the art.

Also provided is a method of screening cells which can be promoted to become an ES cell comprising contacting the cells with basic FGF, LIF, membrane associated SF, and soluble SF in amounts to enhance the growth of and allow proliferation of the cells and determining which cells become ES cells. Utilizing this method, cells other than primordial germ cells and embryonic ectoderm cells can be selected as a source of ES cells.

Since the invention provides ES cells generated for virtually any animal, the invention provides a method of using the ES cells to contribute to chimeras in vivo comprising injecting the cell into a blastocyst and growing the blastocyst in a foster mother. Alternatively, aggregating the cell with a morula stage embryo and growing the embryo in a foster mother can be used to produce a chimera. As discussed above, the ES cells can be manipulated to produce a desired effect in the chimeric animal. The methods of producing such chimeric animals are well established (Robertson (1987)).

Alternatively, the ES cells can be used to derive cells for therapy to treat an abnormal condition. For example, derivatives of human ES cells could be placed in the brain to treat a neurodegenerative disease.

FGF, SF and LIF have been shown herein to be critical for making ES cells. However, as noted above for FGF, other members of the respective growth factor family could also be used to make ES cells. Thus, later discovered members of each family can merely be substituted to determine if the new factor enhances the growth and allows the continued proliferation of PGCs or embryonic ectoderm cells to form ES cells. For example, if a new member of the LIF family is discovered, the new LIF is merely combined with SF and FGF to determine if the new family member enhances the growth and allows the continued proliferation of PGCs or embryonic ectoderm cells. Thus, this invention provides the use of family members and a method of screening family members for activity.

Likewise, additional growth factors may be found useful in enhancing the growth and proliferation of PGCs or embryonic ectoderm cells from various animals. This invention provides combining FGF, SF and LIF with other growth factors to obtain or enhance the production of ES cells. Thus, a method of screening other growth factors for the ability to promote PGCs and embryonic ectoderm cells to form ES cells is also provided. In this regard, IL-11 and IL-6 are good screening candidates and can be used to promote ES cell formation.

EXAMPLES

All the cell types and other materials listed below can be obtained through available sources and/or through routine methods.

MATERIALS AND METHODS

Feeder cells

The Sl/Sl^4 cell line, derived from a homozygous null Sl/Sl mouse embryo, and its derivative, Sl^4-m220, which stably expresses only membrane bound murine SF lacking exon 6 encoding the proteolytic cleavage site, were obtained from Dr. David Williams (Howard Hughes Medical Institute, Indiana University Medical School). Other cell lines which produce adequate SF can be substituted for Sl/Sl^4. They were maintained in DMEM with 10% calf serum and 50 ug/ml gentamicin. For making feeder layers they were irradiated (500 rads) and plated at a density of 2×105 per well of 24-well plates (Falcon) in the same medium, 24 hrs before use. Wells were pre-treated with 1% gelatin. STO cells stably transfected with human LIF and the bacterial neor gene (SLN) were obtained from Dr. Allan Bradley.

Primary cultures of PGCs

Embryos were from ICR females mated with (C57BLxDBA) F1 males. Noon of the day of plug is 0.5 day post coitum (p.c.). The caudal region of 8.5 day p.c. embryos (between the last somite and the base of the allantois) was dissociated into single cells by incubation at 37° C. with 0.05% trypsin, 0.02% EDTA in Ca++/Mg++ free Dulbecco's phosphate-buffered saline (PBS) for about 10 mins with gentle pipetting. At this stage there are between about 149 and 379 PGCs in each embryo (Mintz and Russell, 1957). Cells from the equivalent of 0.5 embryo were seeded into a well containing feeder cells as above and 1 ml of DMEM, 2 mM glutamine, 1 mM sodium pyruvate, 100 i.u./ml penicillin and 100 ug/ml streptomycin and 15% fetal bovine serum (PGC culture medium). Finely minced fragments of genital ridges from 1.5 and 12.5 day p.c. embryos were trypsinized as above and plated at a concentration of 0.1 embryo per well. Growth factors were added at the time of seeding, usually at the following concentrations, which were shown to be optimal for PGC proliferation; recombinant human LIF and bFGF (10–20 ng/ml) and soluble rat SF (60 ng/ml). The medium was changed every day.

Secondary culture of PGC

Primary cultures were trypsinized and reseeded into wells containing Sl^4-m220 feeder layers in PGC culture medium. For further subculture, rounded colonies of densely packed ES-like cells were carefully picked up in a finely drawn pipette and trypsinized in a microdrop under mineral oil before seeding into wells containing feeder cells as above. After several subcultures in this way, cultures were passaged without picking individual colonies.

Alkaline phosphatase (AP) staining

This was carried out as described (Matsui et al. 1991). After staining, AP positive cells were counted using an inverted microscope.

SSEA-1 staining

PGC cultures on Sl^4-m220 feeder cells on a chamber slide (Nunc) were washed twice with PBS containing 2% calf serum, 0.1% sodium azide and then incubated with mouse monoclonal antibody SSEA-1 (1:100 dilution) on ice for 30 min. After washing with PBS, cells were incubated for 30 mins with FITC-conjugated Fab' fragment of goat anti mouse IgG (H+L) (Cappell, 1:5 dilution). After washing in PBS, cells were fixed in 4% paraformaldehyde before staining for AP.

Tumors in nude mice

Approximately 2×106 cells from three independent lines were injected subcutaneously into nude mice (three mice per

line). After three weeks tumors were fixed in Bouin's fixative, processed for histology and sections stained with haematoxylin and eosin.

Chimera formation

Ten to fifteen cells from two independent lines derived from 8.5 day p.c. embryos were injected into the blastocoel of 3.5 day p.c. blastocysts of either ICR or C57BL/6 mice. These were returned to the uteri of 2.5 day p.c. pseudopregnant foster mothers.

Culture of murine PGCs in the presence of growth factors

Initial experiments used Sl/Sl^4 cells derived from a homozygous null Sl/Sl mutant mouse as a feeder layer for the culture of cells dissociated from the posterior of 8.5 days p.c. embryos, and AP staining as a marker for PGCs (FIG. 1A). As shown previously (Matsui et al., 1991), soluble SF and LIF act synergistically on PGCs. Addition of bFGF further enhances growth, and the cells continue to increase in number until day 5 in culture, i.e. one day longer than usual. The effect of bFGF alone is small, and both SF and LIF are needed in addition to bFGF for maximal effect on PGC growth (FIG. 1A, B). A variety of other growth factors, including human activin, Bone Morphogenetic Protein-4, βNGF, and PDGF at 10 and 50 ng/ml had no effect in the presence of SF and LIF.

Membrane associated SF seems to play an important role in PGC proliferation since Sl^d mouse mutants which make only soluble SF have a reduced number of PGCs in vivo, and membrane associated SF is more effective than soluble SF in supporting PGC growth and survival in culture (Dolci et al., 1991; Matsui et al., 1991). To test the effect of added factors in the presence of membrane associated SF, 8.5 day p.c. PGCs were cultured on Sl^4-m220 feeder cells, which express only membrane associated SF (Matsui et al., 1991; Toksoz et al., 1991). Both LIF and bFGF separately enhance PGC growth on Sl^4-m220 feeder cells with added soluble rSF. However, when LIF and bFGF are added together, PGC growth is dramatically stimulated and the cells continue to proliferate through to day 6 in culture (FIG. 1C). The cells survive until day 8, at which time the feeder layer deteriorates, but they can be trypsinized and subcultured (see below).

Pregonadal PGCs are motile in vivo, and when cultured with LIF on a Sl^4-m220 feeder layer they form burst colonies of cells with a flattened and polarized morphology, characteristic of motile cells (FIG. 2A). In contrast, PGCs cultured on a Sl^4-m220 feeder layer with soluble SF and bFGF or with bFGF and LIF (FIGS. 2B, C), form discrete colonies of tightly packed cells. These colonies increase in size over day 6 in culture only when both bFGF and LIF are present (FIG. 2C).

To determine whether PGCs and their descendants continue to proliferate in culture, primary colonies of PGCs were trypsinized after 6 days in culture and replated on a fresh Sl^4-m220 feeder layer with added growth factors. By day 6 in secondary culture, large colonies of densely packed AP positive cells resembling embryonic stem (ES) cells are present (FIGS. 2D, E; FIGS. 4, A), with an overall plating efficiency of about 5%. These colonies are also positive for the expression of the antigen SSEA-1, a characteristic of PGCs (Donovan et al., 1986) and undifferentiated embryonal carcinoma and ES cells (Solter and Knowles, 1978) (FIGS. 2G, H). Although the growth of primary cultures is strictly dependent on the presence of LIF and bFGF, secondary colonies can form in the absence of these factors (Table 1), indicating a reduced exogenous growth factor requirement for the descendants of PGCs after subculture. Most of the colonies show strong, uniform AP staining. However, some colonies contain only a small number of strongly stained cells, surrounded by cells which are weakly stained or negative (FIG. 2E). In many cases these negative cells are larger and have a more flattened morphology than the AP positive cells. For further subculture, individual colonies of cells with a distinctive, tightly packed, ES cell-like morphology were picked up in a micropipet, trypsinized and replated on a fresh feeder layer with added factors. Such colonies can be subcultured at least ten times and continue to give rise to colonies of similar morphology. In later passages, these cultures were transferred to feeder layers of STO cells in medium without added factors normally used for blastocyst-derived ES cell culture (Robertson, 1987). Under these conditions they continue to proliferate in an undifferentiated state, for a total of at least 20 passages.

Two independent lines at passage 14 (1/14, 2/14) and one at passage 20 (3/20) were karyotyped. Most cells had a normal or near normal XY karyotype, but in two lines (2/14 and 3/20) there was a significant proportion of trisomic cells.

Long term culture of PGC-derived cells from genital ridges

Since transplantable teratocarcinomas can be induced experimentally by grafting genital ridges from 11.5 or 12.5 days p.c. male embryos of the 129 strain to an ectopic site, we tested the possibility that ES-like cells can be obtained from genital ridges in culture. Genital ridges were trypsinized and the cells plated on an Sl^4-m220 feeder layer with soluble SF, LIF and bFGF. The number of PGCs initially declines but increases after 3 days, and by 6 days colonies of densely packed, AP positive cells can be seen (FIG. 2F). If cells from male and female 12.5 days p.c. genital ridges are cultured separately, male PGCs increase in number and form colonies. In contrast, only a few female PGCs form colonies (FIG. 3). The differentiation capacity of genital ridge-derived colonies has not so far been tested.

Differentiation of PGC-derived ES cells in vitro and in nude mice

Four independent lines of undifferentiated cells derived from 8.5 day embryos and cultured onto STO feeder layers were trypsinized and pipetted gently to generate small clumps of cells which were then placed in bacteriological plastic dishes. After five to seven days most of the clumps differentiated into typical simple or cystic embryoid bodies (EBs), with a clear outer layer of extraembryonic endoderm cells (FIGS. 4, B). When these EBs were returned to tissue culture plastic dishes they rapidly attached and over two weeks gave rise to a variety of cell types, including extraembryonic endoderm, spontaneously contracting muscle, nerve and endothelial and fibroblast-like cells.

Three of these four lines, at passages 9 and 15 on STO cells, were injected subcutaneously into nude mice. Each line gave rise to multiple, well-differentiated teratocarcinomas, containing a wide variety of tissues, including keratinized, secretory and ciliated epithelium, neuroepithelium and pigmented epithelium, cartilage, bone, and muscle, as well as nests of undifferentiated embryonic cells (FIGS. 4, C–E).

PGC-derived ES cells can contribute to chimeras in vivo

To test whether the descendants of PGCs in culture are able to contribute to chimeras in vivo, 10–15 cells with an ES-like morphology from two independent early passage cultures derived from 8.5 day embryos and cultured on either Sl^{4}m220 cells or STO cells were injected into host ICR or C57BL/6 blastocysts. From a total of 21 pups born, four were chimeric, as judged by coat color, but only two were extensive, with approximately 50 and 90% chimerism. The 50% coat color chimera, generated by injecting cells

from the 4th passage on STO cells into an ICR blastocyst, died at 11 days after birth and showed stunted growth and skeletal abnormalities. The 90% coat color chimera, obtained by injecting cells from the 6th passage on STO cells into a C57BL/6 blastocyst, had no obvious abnormalities.

Generation of ES cells from other mammals

ES cells from other mammals can be produced using the methods described above for murine. The mammalian cell of choice is simply substituted for murine and the murine methods are duplicated. The appropriate species specific growth factors (e.g. SF) can be substituted for murine growth factors as is necessary. Any additional growth factors which can promote the formation of ES cells can be determined by adding the growth factors to FGF, LIF, and SF as described above and monitored for an affect on ES formation.

Generation of chimeras using non-murine ES cells

Chimeras utilizing non-murine ES cells can likewise be produced utilizing the methods for murine described above and simply substituting the appropriate non-murine blastocyst for the species of ES utilized.

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The preceding examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may be alternatively employed.

TABLE 1

	Growth Factor Requirements for Secondary Cultures of PCG-Derived Cells		
Days in Culture	SF→SF+LIF+LIF+ bFGF	SF+LIF+bFGF→SF+ LIF+bFGF	SF+ LIF+bFGF→SF
1	112 ± 16 cells	116 ± 20 cells	142 ± 18 cells
3	0.9 ± 0.6 colonies	4.6 ± 1.1 colonies	5.6 ± 0.8 colonies
5	0.5 ± 0.4 colonies	6.9 ± 1.2 colonies	6.6 ± 1.3 colonies

PGCs from 8.5 dpc embryos were cultured for 6 days on Sl^4-m220 cells in the presence of either soluble rat SF alone or with soluble rat SF, LIF, and bFGF. Cultures were trypsinized and seeded into wells containing Sl^4-m220 feeder cells with either soluble rat SF alone or soluble rat SF, LIF, and bFGF. Cultures were fixed and AP-positive cells (day 1) or colonies (days 2 and 5) counted. Numbers are mean ± SEM from four experiments. Secondary cultures show a reduced growth factor requirement compared with primary cultures.

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What is claimed is:

1. A composition comprising:

(a) pluripotential embryonic stem cells; and

(b) a fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor in amounts to enhance the growth of and allow the continued proliferation of the cells.

2. The composition of claim 1, wherein the fibroblast growth factor is basic fibroblast growth factor.

3. A composition comprising:

(a) primordial germ cells; and

(b) a fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor and soluble steel factor in amounts to enhance the growth of and allow the continued proliferation of the cells.

4. The composition of claim 3, wherein the fibroblast growth factor is basic fibroblast growth factor.

5. A composition comprising:

(a) embryonic ectoderm cells; and

(b) fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor and soluble steel factor in amounts to enhance the growth of and allow the continued proliferation of the cells.

6. A composition comprising a fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor in amounts to enhance the growth of and allow the continued proliferation of primordial germ cells.

7. The composition of claim 6, wherein the fibroblast growth factor is basic fibroblast growth factor.

8. A method of making a pluripotential embryonic stem cell comprising administering a growth enhancing amount of basic fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor to primordial germ cells under cell growth conditions, thereby making a pluripotential embryonic stem cell.

9. The method of claim 8 wherein the stem cell is derived from a mammal.

10. The method of claim 9, wherein the mammal is a mouse.

11. A method of making a pluripotential embryonic stem cell comprising administering a growth enhancing amount of basic fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor to embryonic ectoderm cells under cell growth conditions, thereby making a pluripotential embryonic stem cell.

12. The method of claim 11, wherein the stem cell is derived from a mammal.

13. A method of screening cells which can be promoted to become pluripotent embryonic stem cells comprising contacting the cells with basic fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor in amounts to enhance the growth of and allow proliferation of the cells and determining which cells become pluripotent embryonic stem cells.

14. A composition comprising a fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor in amounts to promote the formation of pluripotent embryonic stem cells from primordial germ cells.

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